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Molecular Characterization of a Novel Glucosyltransferase from *Lactobacillus reuteri* Strain 121 Synthesizing a Unique, Highly Branched Glucan with α -(1→4) and α -(1→6) Glucosidic Bonds

S. Kralj,^{1,2} G. H. van Geel-Schutten,^{1,3} H. Rahaoui,⁴ R. J. Leer,³ E. J. Faber,⁵
M. J. E. C. van der Maarel,^{1,6} and L. Dijkhuizen^{1,2*}

Centre for Carbohydrate Bioengineering, TNORUG,¹ and Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren,² Department of Applied Microbiology and Gene Technology³ and Department of Food Microbiology TNO Nutrition and Food Research,⁴ Zeist, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, Utrecht,⁵ and Carbohydrate Technology Department, TNO Nutrition and Food Research, Groningen,⁶ The Netherlands

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Lactobacillus reuteri strain 121 produces a unique, highly branched, soluble glucan in which the majority of the linkages are of the α -(1→4) glucosidic type. The glucan also contains α -(1→6)-linked glucosyl units and 4,6-disubstituted α -glucosyl units at the branching points. Using degenerate primers, based on the amino acid sequences of conserved regions from known glucosyltransferase (*gtf*) genes from lactic acid bacteria, the *L. reuteri* strain 121 glucosyltransferase gene (*gtfA*) was isolated. The *gtfA* open reading frame (ORF) was 5,343 bp, and it encodes a protein of 1,781 amino acids with a deduced M_r of 198,637. The deduced amino acid sequence of GTFA revealed clear similarities with other glucosyltransferases. GTFA has a relatively large variable N-terminal domain (702 amino acids) with five unique repeats and a relatively short C-terminal domain (267 amino acids). The *gtfA* gene was expressed in *Escherichia coli*, yielding an active GTFA enzyme. With respect to binding type and size distribution, the recombinant GTFA enzyme and the *L. reuteri* strain 121 culture supernatants synthesized identical glucan polymers. Furthermore, the deduced amino acid sequence of the *gtfA* ORF and the N-terminal amino acid sequence of the glucosyltransferase isolated from culture supernatants of *L. reuteri* strain 121 were the same. GTFA is thus responsible for the synthesis of the unique glucan polymer in *L. reuteri* strain 121. This is the first report on the molecular characterization of a glucosyltransferase from a *Lactobacillus* strain.

Many lactic acid bacteria employ large extracellular enzymes, glucosyltransferases (GTFs) (EC 2.4.1.5; common name, glucansucrases), for the synthesis of high-molecular-weight α -glucans from sucrose. Moreover, low-molecular-weight oligosaccharides are produced in the presence of suitable acceptor molecules.

The GTF enzymes of oral streptococci and the dextranases (DSRs) and alternansucrases (ASRs) from *Leuconostoc mesenteroides* strains have been studied in the most detail. All GTFs from lactic acid bacteria share a common structure and are composed of four distinct domains: their N-terminal end starts with (i) a signal peptide of 32 to 34 amino acids, which is followed by (ii) a highly variable stretch of 123 to 129 amino acids, (iii) a highly conserved catalytic or sucrose binding domain of about 1,000 amino acids, and (iv) a C-terminal glucan binding domain of about 500 amino acids, which is composed of a series of tandem repeats (30).

Amino acid sequence comparisons revealed that GTFs possess a $(\beta/\alpha)_8$ barrel structure similar to that of glycoside hy-

drolases of family 13. This family includes, for instance, α -amylase and cyclodextrin glycosyltransferase (CGTase) (43). The core of the proteins belonging to this family comprises eight β -sheets alternated with eight α -helices. In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (12, 27). Therefore, GTFs are classified in family 70 of glycoside hydrolases (<http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). Some GTF enzymes, such as dextranase from *L. mesenteroides* strain B-512F, catalyze the formation of linear glucans containing mostly α -(1→6) linkages (dextrans). Other types synthesize dextrans with α -(1→2), α -(1→3), or α -(1→4) branches (16, 30). ASR from *L. mesenteroides* strain NRRL B-1355 synthesizes a glucan with alternating α -(1→6) and α -(1→3) glucosidic bonds (3). Recently, a GTF of *L. mesenteroides* strain NRRL B-1355 that synthesizes a glucan containing α -(1→2) glucosidic linkages has been characterized (39). There are few reports, however, about glucan synthesis in lactobacilli (13, 21, 37, 44, 45). A biochemical and molecular characterization of the enzyme(s) responsible for glucan synthesis in lactobacilli has not been reported.

In previous studies we have isolated a strain of *Lactobacillus reuteri* that is capable of producing both a fructan and a glucan. Depending on the carbon source in the culture medium, the GTF responsible for the synthesis of this polysaccharide ma-

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands. Phone: 31.50.3632150. Fax: 31.50.3632154. E-mail: L.Dijkhuizen@biol.rug.nl.

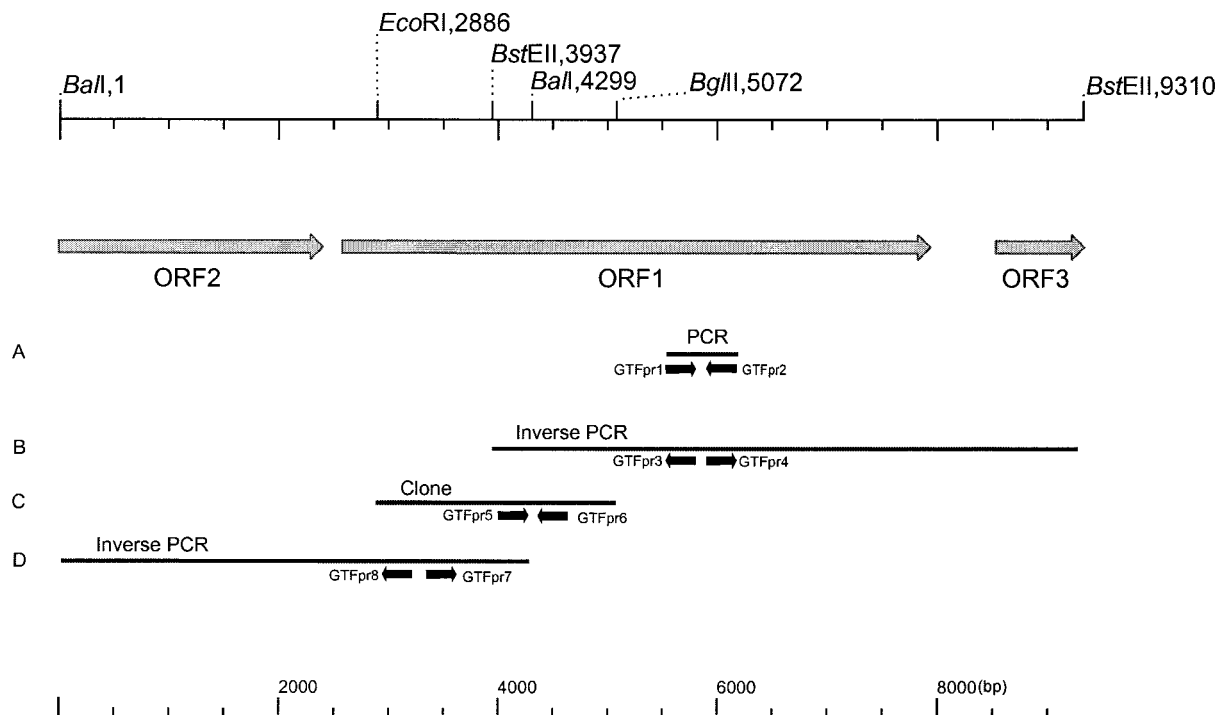


FIG. 1. Strategy used for the isolation of the *gtfA* gene and surrounding regions from *L. reuteri* strain 121 chromosomal DNA. Primers are indicated with small, black arrows. (A) The fragment given is the 659-bp insert isolated with degenerated primers. (B and D) The fragments are regions amplified by inverse PCR (5,229 and 4,229 bp, respectively). (C) The fragment (2,186 bp) was obtained by colony blot hybridization of a partial gene library.

terial is completely cell associated or partly released into the culture medium (44, 45). This work describes the first molecular characterization of a *Lactobacillus* GTF gene (*gtfA*), expression of *gtfA* in *Escherichia coli*, and the characterization of its glucan product. The *gtfA* gene encodes a novel type of GTF, synthesizing a highly branched glucan with a unique structure containing α -(1 \rightarrow 4) and α -(1 \rightarrow 6) linkages.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *L. reuteri* strains 121 and 35-5 (both obtained from the culture collection of TNO Nutrition and Food Research, Zeist, The Netherlands) were cultivated anaerobically at 37°C in MRS medium (Difco, Franklin Lakes, N.J.) (9) or in MRS-s medium (i.e., MRS medium with sucrose [100 g liter⁻¹] instead of glucose [20 g liter⁻¹]). *E. coli* DH5 α (Phabagen, Utrecht, The Netherlands) (22), *E. coli* JM 109 (Promega, Madison, Wis.) (22), and *E. coli* TOP 10 (Invitrogen, Carlsbad, Calif.) were used as hosts for cloning purposes. Plasmids pCR2.1-TOPO (Invitrogen), pCR-XL-TOPO (Invitrogen), and pEMBL8 (11) were used for cloning of the GTF gene for sequencing purposes. Plasmid pBluescript II SK(+) (Stratagene, La Jolla, Calif.) was used for cloning of the complete GTF gene. Plasmid pET15b (Novagen, Madison, Wis.) was used for expression of the *gtf* gene in *E. coli* BL21 Star(DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37°C in LB medium (4). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (ampicillin [100 μ g ml⁻¹] or kanamycin [50 μ g ml⁻¹]). Agar plates were made by adding 1.5% agar to the LB medium; when appropriate, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) (20 μ g ml⁻¹) was added.

Isolation of DNA. *L. reuteri* strain 121 total DNA was isolated according to the method in reference 32. Plasmid DNA of *L. reuteri* strain 121 was isolated using a modification of the methods described previously (2, 7). Prewarmed (37°C) fresh MRS broth (10 ml) was inoculated with 200 μ l of an overnight culture and incubated for 2.5 h at 37°C. Cells were harvested by centrifugation and washed with 2 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH

8]). After centrifugation, the pellet was resuspended in 380 μ l of solution I (0.5 M sucrose, 50 mM Tris-HCl, 1 mM EDTA [pH 8]) containing lysozyme (2 mg/ml; Sigma, St. Louis, Mo.) and 6.6 U of mutanolysin (Sigma). After 1.5 h of incubation at 37°C, 50 μ l of solution II (0.25 M EDTA, 50 mM Tris-HCl [pH 8]) and 30 μ l of solution III (20% sodium dodecyl sulfate, 50 mM Tris-HCl, 20 mM EDTA [pH 8]) were added and the suspension was mixed. NaOH (30 μ l of 3 M solution) was added, which was followed by 50 μ l of 2 M Tris-HCl and 72 μ l of 5 N NaCl. After extraction with equal volumes of phenol and chloroform, the DNA was precipitated with ethanol as described previously (35).

Plasmid DNA of *E. coli* was isolated using the alkaline lysis method (6) or with a Wizard Plus SV plasmid extraction kit (Promega).

Molecular techniques. General procedures for cloning, *E. coli* transformations, DNA manipulations, and agarose gel electrophoresis were carried out as described previously (35). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs, Beverly, Mass.; Roche Biochemicals, Basel, Switzerland). Primers were obtained from Eurogentec, Seraing, Belgium. Sequencing was performed according to the method in reference 36. DNA was amplified by PCR on a DNA Thermal Cycler 480 (Perkin-Elmer, Boston, Mass.) using *ampliTAQ* DNA polymerase (Perkin-Elmer) or *Pwo* DNA polymerase (Roche Biochemicals). For inverse PCR the Expand High Fidelity PCR system (Roche Biochemicals) was used as described by the supplier. Fragments were isolated from agarose gels using a Qiagen (Hilden, Germany) gel extraction kit following the instructions of the supplier.

For Southern hybridization, DNA was restricted with endonucleases, separated by agarose gel electrophoresis, and transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) following the manufacturer's instructions. The probes, which were labeled with digoxigenin-dUTP, were prepared, and patterns of hybridization with the probes were examined with a digoxigenin DNA labeling and detection kit (Roche Biochemicals), following the manufacturer's instructions. Stringent probe hybridizations were performed at 65°C, and nonstringent hybridizations were performed at 45°C.

Identification and nucleotide sequence analysis of the GTF gene. An overview of the isolation strategy of the *gtf* gene is given in Fig. 1. The first part of the GTF gene was isolated by amplification of chromosomal DNA of *L. reuteri* strain 121

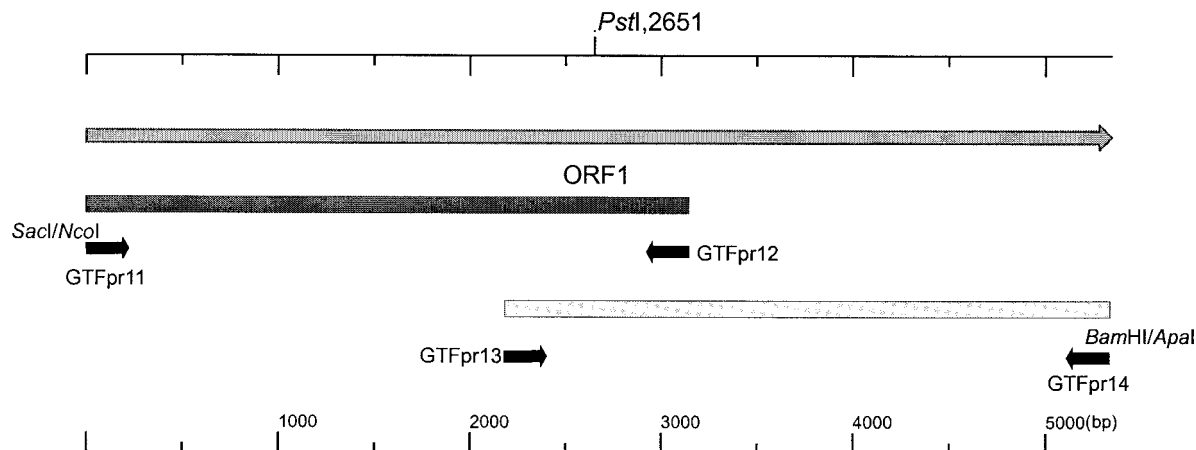


FIG. 2. Overview of primers and restriction sites used for cloning of the *gtfA* gene in the expression vector pET15B (Novagen).

with PCR using degenerate primers (GTFpr1, 5'-GAYAAKWSNAKSYNRT NGTNSARGC-3', and GTFpr2, 5'-ANRTNCNCRTARTANACNCKNG-3' [Y = T or C; K = G or T; W = A or T; S = C or G; R = A or G; N = A, C, G, or T]) based on conserved amino acid sequences present in the catalytic core, as deduced from the GTF genes of *Streptococcus downei* (*gtfS*), *Streptococcus mutans* (*gtfC*), *S. downei* (*gtfT*), *Streptococcus salivarius* (*gtfK* and *gtfM*), and *dsrA* of *L. mesenteroides* (14, 18, 20, 31, 38, 40). An amplification product with the expected size of about 660 bp was obtained and cloned in *E. coli* JM 109 using the pCR2.1 vector. Analysis of its nucleotide sequence (659 bp [Fig. 1A]) confirmed its GTF identity. The 659-bp amplified fragment was used to design the primers GTFpr3 (5'-GGGCTTTCAGATGCAACTAATCGTTGGGG-3') and GTFpr4 (5'-GCTTATTTGAGACAGCATCTGAGTCCATACC-3') for inverse PCR. *L. reuteri* strain 121 chromosomal DNA was digested with *Bst*EII and ligated, yielding circular DNA molecules. PCR with diverging primers GTFpr3 and GTFpr4 with the circular ligation products as template yielded an amplicon of 5,229 bp (Fig. 1B), which was cloned into pCR-XL-TOPO (Invitrogen). Based on the 5,229-bp nucleotide sequence, a probe encompassing GTFpr5 (5'-GGT AATGATAAGCGCCATACAG-3') and GTFpr6 (5'-GATATACTGCTGCTA CATCTGGAC-3') was designed to screen a partial gene library of *L. reuteri* strain 121. This library was constructed by ligating *Eco*RI/*Bgl*II fragments of genomic DNA into the *Eco*RI/*Bam*HI sites of pEMBL8. A positive clone was selected by colony blot hybridization (8) using Hybond N-filters (Amersham Pharmacia Biotech), and its nucleotide sequence was determined (2,186 bp [Fig. 1C]). This information was used to design the diverging primers GTFpr7 (5'-C GGGATTCAATTGAAATTGTTAGTCGATACAG-3') and GTFpr8 (5'-GAG ATCGCTTCGTTGCATTCATCCTGAAAC-3') for an inverse PCR step with *Bal*I-digested and ligated chromosomal DNA. The inverse PCR fragment obtained was cloned into pCR-XL-TOPO (Invitrogen) and sequenced (4,299 bp [Fig. 1D]).

Construction of plasmids for expression of the GTF gene in *E. coli*. Two separate PCRs with primers GTFpr11 to -14 were used to amplify the complete *gtf* gene (Fig. 2). The product of the first PCR, synthesized with primers GTFpr11 (5'-GATGCATGAGCTCCATGGACCAACAAGTTCAGCAAGC TTCC-3', containing *Sac*I [in boldface type] and *Nco*I [underlined] sites) and GTFpr12 (5'-GTGCATTAAAGTACGTAACCAATCAGTATTTCCGG-3'), was digested with *Sac*I/*Pst*I. The product of the second PCR, primed with oligonucleotides GTFpr13 (5'-TTGATGGTATGGTGGCCTAATACTCTTAC CC) and GTFpr14 (5'-ATATCGATGGGCCCGGATCCTATTAGTGATGGT GATGGTGATGTAGTTTATTTTATGCAAGCATCTTACC-3', containing *Apa*I [in boldface type] and *Bam*HI [underlined] sites and a six-His tag [in italics]), was digested with *Pst*I/*Apa*I. The resulting fragments (2,547 and 2,723 bp) were cloned in the corresponding sites of pBluescript II SK(+), yielding pBSP2500 and pBPA2700, respectively. Both plasmids were digested with *Apa*I/*Pst*I, and the fragment from pBPA2700 containing the 3' part of *gtf* was ligated into pBSP2500, yielding pBGTF1. Plasmid pBGTF1 was digested with *Nco*I/*Bam*HI, and the resulting 5.3-kb fragment was ligated into the corresponding sites of the expression vector pET15b (Novagen) yielding p15gtf.

Dendrogram construction. Amino acid sequences were aligned with Clustal W 1.74 (23), with a gap opening penalty of 30 and a gap extension penalty of 0.5. Amino acid sequences were obtained from GenBank (accession numbers are

given in parentheses) and divided into the following three groups: (i) DSRB of *L. mesenteroides* NRRL B-1299 (AAB95453), DSRS of *L. mesenteroides* NRRL B-512F (AAA53749), DSRA of *L. mesenteroides* NRRL B-1299 (AAB40875), ASR of *L. mesenteroides* NRRL B-1355 (CAB65910), DSRT of *L. mesenteroides* NRRL B-512F (BAA90527), and GTFA of *L. reuteri* strain 121; (ii) GTFIa of *Streptococcus sobrinus* OMZ176 (BAA14241), GTFI of *S. downei* Mfe28 (BAA0296), GTFIs of *S. sobrinus* OMZ176 (BAA02976), GTFB of *S. mutans* GS5 (AAA88588), and GTFC of *S. mutans* GS5 (AAA88589); and (iii) GTFG of *Streptococcus gordonii* (AAC43483), GTFR of *Streptococcus oralis* ATCC 10557 (BAA95201), GTFs of *S. downei* Mfe28 (AAA26898), GTFM of *S. salivarius* ATCC 25975 (AAC41413), GTFL of *S. salivarius* ATCC 25975 (AAC41412), GTFN of *S. salivarius* ATCC 25975 (AAC05165), GTFD of *S. mutans* GS5 (AAA26895), GTFJ of *S. salivarius* ATCC 25975 (CAA77900), GTFI of *S. sobrinus* OMZ176 (D13928), and GTFK of *S. salivarius* ATCC 25975 (CAA77898). Amino acid sequences were aligned first within each group. The complete alignment was performed by aligning groups i to iii with each other. Tree construction was performed using TreeCon 1.3b (no correction for distance estimation, 10 bootstrap samples, and the neighbor-joining algorithm were used) (42).

Preparation of *E. coli* cell extracts. Cells of *E. coli* BL21 Star(DE3) harboring p15gtf were harvested by centrifugation (10 min at 4°C at 10,000 × g) after 16 h of growth. The pellet was washed with 50 mM sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂ and 1% (vol/vol) Tween 80, and the suspension was centrifuged again (10 min at 4°C at 10,000 × g). Pelleted cells were resuspended in 50 mM sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂, 1% (vol/vol) Tween 80, and 5 mM β-mercaptoethanol. Cells were broken by sonication (seven 15-s pulses of 7 μ at 30-s intervals). Cell debris and intact cells were removed by centrifugation for 20 min at 4°C at 10,000 × g, and the resulting cell extract (supernatant) was used in the enzyme assays.

Enzyme assays. Using *E. coli* cell extracts or *L. reuteri* strain 121 grown on MRS-s culture supernatant as the source of enzyme, GTF activity was measured by determining the release of fructose from sucrose at 37°C in 50 mM sodium acetate buffer, pH 5.5, containing 1 mM CaCl₂ and 100 mM sucrose (44).

Amino acid sequence determination from the GTF from *L. reuteri* strain 121 using 2D-PAGE. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (i.e., isoelectric focusing followed by sodium dodecyl sulfate-PAGE) experiments were performed as described earlier (33), using the II xi Cell system according to the manufacturer's instructions (Bio-Rad, Hercules, Calif.). Chemicals and materials were from Bio-Rad, unless indicated otherwise.

(i) **Sample preparation.** Supernatants of cultures of *L. reuteri* strain 121 grown on MRS-s (10 ml precipitated with 2 ml of 30% trichloroacetic acid) in the exponential phase of growth, harvested by centrifugation (10,000 × g at 4°C for 10 min), were used as a source of GTF protein.

(ii) **GTF activity staining.** After separation in the second dimension, GTF activity was identified as described previously (44), using periodic acid-Schiff staining. The same gel was silver stained (Genotech, St. Louis, Mo.) to identify other proteins on the gel, thereby facilitating the identification of the GTF spot on the blot (see below).

(iii) **Amino acid sequencing.** A second gel obtained after 2D-PAGE, identical to the GTF activity stained gel, was blotted according to the method in reference

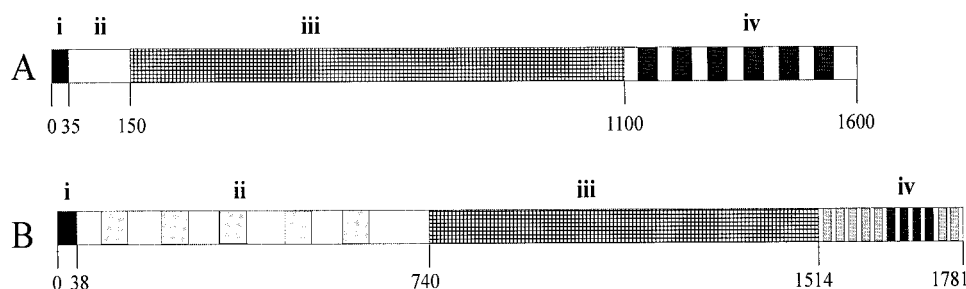


FIG. 3. Schematic representation of GTFs in general (A) and GTFA of *L. reuteri* strain 121 (B), showing the four different domains—(i) N-terminal signal sequence, (ii) variable region with five RDV repeats (light grey boxes), (iii) catalytic domain, and (iv) C-terminal (putative) glucan binding domain with four YG repeating units (dark grey boxes)—according to the definition of Giffard and Jacques (19) and seven less-conserved YG repeating units (light grey boxes).

26 onto a polyvinylidene difluoride membrane (Roche Biochemicals) with a semidry electroblotter (Ancos, Hoejby, Denmark). The polyvinylidene difluoride membrane was stained with Coomassie brilliant blue R-250. The N-terminal amino acid sequence of the protein band which corresponded with the activity band obtained by periodic acid-Schiff staining was determined on a Perkin-Elmer ABI 476A automated sequencer using Edman degradation (NAPS Protein Sequencing and Peptide Mapping Laboratory, Vancouver, Canada).

Characterization of the glucans produced. Glucans were produced by incubating the enzyme preparations overnight, using the conditions described above under "Enzyme assays." Glucans produced by *L. reuteri* strain 121 and strain 35-5 (producing only the wild-type glucan and not the fructan) and glucans produced with the GTF expressed in *E. coli* were isolated by precipitation with ethanol (44). Nuclear magnetic resonance (NMR) spectroscopy and methylation analysis were performed as described earlier (44). The molecular weights of the glucans were determined by high-performance size exclusion chromatography (HPSEC) coupled online with a multiangle laser light scattering (MALLS) and a differential refractive index detection (Schambeck SDF). The HPSEC system consisted of an isocratic pump, an injection valve, a guard column, and a set of two SEC columns in series (Shodex SB806MHQ column and TSK gel 6000PW). A Dawn-DSP-F (Wyatt Technology, St. Barbara, Calif.) laser photometer (HeNe [$\lambda = 632.8$ nm]) equipped with a K5 flow cell, thermostatted by a Peltier heating system, was used as a MALLS detector. Samples were filtered through a 0.45- μ m-pore-size filter (MILLEX), and the injection volume was 220 μ l. Na_2SO_4 (0.1 M) was used as eluent at a flow rate of 0.8 ml min^{-1} . Pullulan and dextran samples with M_w ranging from 4×10^4 to 2×10^6 were used as standards. Determinations were performed in duplicate.

Nucleotide accession number. The nucleotide sequence of *gtfA* has been assigned accession no. AX306822 by GenBank.

RESULTS

Isolation and nucleotide sequence analysis of the putative *L. reuteri* strain 121 GTF gene. Based on sequence homology between conserved regions located in the catalytic core of different *gtf* genes of gram-positive bacteria, degenerate primers were designed and used for PCR with chromosomal DNA of *L. reuteri* strain 121 as the template. A single fragment of 659 bp (Fig. 1) was obtained, and sequence analysis confirmed its *gtf* identity. Southern hybridization of chromosomal DNA of *L. reuteri* strain 121 with this 659-bp amplified PCR fragment, followed by washing under nonstringent conditions, revealed one hybridizing fragment. Plasmid DNA of *L. reuteri* did not show hybridization.

In subsequent steps a total of 9,310 bp was obtained and sequenced (Fig. 1). One complete open reading frame (ORF) and two partial ORFs were located on this compiled sequence: ORF1 (5,343 bp [Fig. 1]), which encodes a putative GTF (GTFA); ORF2 (2,403 bp), upstream of ORF1; and ORF3 (786 bp), downstream of *gtfA*. The deduced amino acid sequence of ORF2 showed homology with GTFs, whereas the

deduced amino acid sequence of ORF3 did not show significant homology to any protein present in databases. The *gtfA* gene encodes a putative protein of 1,781 amino acids, with a deduced molecular weight of 198,637 and a pI of 5.04. It was preceded by a putative ribosomal binding site (GAAGGAG), localized 6 bp upstream from the ATG start codon. According to the consensus promoter sequences described previously for lactobacilli (34), a potential promoter sequence in the sequence upstream of *gtfA* (42 bp from the start codon) could be identified, with a -35 sequence (TTGAAA) separated by 19 bp from a -10 sequence (TATAAT).

Two inverted repeats were located 61 bp downstream from the *gtfA* termination codon. These repeats could form a stem (20 bp)-loop (12 nucleotides) secondary structure with a ΔG value of -20.4 kcal mol^{-1} , followed by a series of thymidine residues, suggesting a rho-independent transcription termination signal (5).

Amino acid sequence alignments of *L. reuteri* GTFA with other GTFs. Alignment of the amino acid sequence of GTFA with other GTFs, performed with BLAST (1), revealed clear similarities. The highest similarity (46% identity and 59% similarity within 1,261 amino acids) at the amino acid level was found with ASR from *L. mesenteroides* NRRL B-1355. The putative protein structure of GTFA was similar to that of other GTFs containing (i) an N-terminal signal sequence of 38 amino acids, (ii) a relatively large variable N-terminal domain of 702 amino acids, (iii) a catalytic domain of 774 amino acids, and (iv) a C-terminal domain of 267 amino acids (Fig. 3).

The deduced N-terminal amino acid sequence of GTFA contained a putative secretion peptide with a predicted signal peptidase cleavage site between amino acids 38 and 39 (<http://www.cbs.dtu.dk/services/SignalP/>). To confirm this cleavage site, the *L. reuteri* strain 121 enzyme was purified by 2D-PAGE and subjected to N-terminal sequence analysis. The first 13 amino acids were identified as DQQVQQASTLQDQ; except for the 10th residue the sequence was identical to that of the deduced amino acid sequence following the predicted cleavage site. 2D-PAGE experiments also confirmed the predicted M_r and pI of GTFA. Within the deduced N-terminal variable region of GTFA, a series of five repeating units was found. These repeating units, designated RDV, were on average 41 amino acids long, separated on average by 71 amino acids (Fig. 4). These repeats have never been seen in other GTF enzymes

RDV1	174	--	RPDV KNVH-NVYNADN	SGF	DNVNVNIDFSKMKDYRDSIEIVSRYS	--	224
RDV2	290	--	RPDVA KVYPQVVGAA	SGF	NVTFNI--SDL-DYTHQYQVLSRYS	--	338
RDV3	411	--	RPDVA KAYPTVKTATN	SGF	KVTFKV--NNL-QPGHQYSVVS	--	459
RDV4	528	--	RPDVA AVYPSLYNSAV	SGF	DTTIKLTNDQYQALNGQLQVLLRFS	--	579
RDV5	654	--	RNDV YKVNPAIENSSM	SGF	QGIITLP---VTVKNENVQLVHRFS	--	702
			* ** . : : *** . . . : . . : * : *				

FIG. 4. Alignment of the five unique repeat elements (RDV1 to RDV5) from the N-terminal region of GTFA of *L. reuteri* strain 121. Conserved (.), highly conserved (:), and identical (*) residues are indicated. The RDV motif is indicated in boldface type [R(N/P)DV]. Two other conserved motifs, SGF and R(F/Y)S, are indicated by boxes.

and showed no significant homology to any protein motifs present in the databases.

The putative catalytic domain of GTFA showed high similarity (about 45% identity and 60% similarity) to other known streptococcal and *Leuconostoc* GTF enzymes. However, not all of the conserved amino acids found in the other GTFs were found in the deduced amino acid sequence of GTFA. Particularly in the region downstream of Asp¹⁰²⁴, 3 out of 10 conserved amino acids were not found in GTFA (Fig. 5). One of the conserved amino acid substitutions in this region of GTFA, Ile¹⁰²⁹, was also found in amylosucrase, a GTF from *Neisseria polysaccharea* synthesizing an α -(1 \rightarrow 4) glucan (10).

The relatively short C-terminal domain of GTFA contains four YG-repeating units according to the definition of Giffard and Jacques (19) and seven YG-repeating units which are less conserved (Fig. 6).

Dendrogram. Construction of a dendrogram (Fig. 7), based on the complete amino acid sequences of different GTFs of lactic acid bacteria, revealed that GTFA of *L. reuteri* strain 121 is distinct from other GTFs known. Again, GTFA is most closely related to the ASR of *L. mesenteroides* NRRL B-1355 (3).

Analysis of the glucans produced by *L. reuteri* and *E. coli* containing p15gtf. Extracts of *E. coli*(p15gtf) cells and supernatants of sucrose-grown cultures of *L. reuteri* incubated with sucrose both produced high-molecular-weight glucans. Using HPSEC-MALLS, the average molecular weight of the glucan produced by *L. reuteri* strain 35-5 was determined to be 4×10^7 ($\pm 5\%$ SEM), whereas that of the glucan produced by *E. coli* harboring p15gtf was 8×10^7 ($\pm 5\%$ SEM).

The ¹H NMR spectra of the glucans produced by the recombinant GTF present in *E. coli* and the GTF enzyme in *L. reuteri* strain 35-5, producing only the glucan, were virtually identical (Fig. 8). Comparison of both ¹H NMR spectra with that of potato starch (17) showed that both glucans consist of

α -(1 \rightarrow 4)- and α -(1 \rightarrow 6)-linked glucopyranosyl units. The identical nature of the glucans was confirmed by methylation analysis (Table 1).

DISCUSSION

This work reports the molecular characterization of the first *Lactobacillus* gene (*gtfA*) encoding a GTF enzyme (GTFA). A detailed analysis showed that GTFA produces a unique soluble glucan in which the majority of the linkages are of the α -(1 \rightarrow 4) glucosidic type. The glucan also contains α -(1 \rightarrow 6)-linked glucosyl units and 4,6-disubstituted α -glucosyl units at the branching points. Expression of *gtfA* in *E. coli* yielded an active GTF synthesizing the same highly branched soluble glucan.

GTFA showed high similarity with streptococcal and *Leuconostoc* GTFs. Similar to other GTFs, GTFA contains an N-terminal signal sequence, a variable N-terminal domain, a catalytic core, and a C-terminal domain. Striking features of GTFA are its relatively large variable domain (702 amino acids), which contains five distinct unique repeats (RDV repeats) (Fig. 4), and its relatively short C-terminal domain (267 amino acids). ASR of *L. mesenteroides* NRRL B-1355 also possesses three N-terminal repeats (24), but these do not show homology to the N-terminal repeats found in GTFA. The exact function of the variable domain is unknown. The variable domain of GTFI from *S. downei* MFe28 contains no repeats and is five times smaller than the GTFA domain. Deletion of the GTFI variable domain yielded a mutant enzyme which retained function (28).

Based on alignments with other GTFs from lactic acid bacteria (30), the putative catalytic residues in *L. reuteri* strain 121 GTFA are Asp¹⁰²⁴, Glu¹⁰⁶¹, and Asp¹¹³³. The putative calcium-binding site is Asp⁹⁸⁶, and the putative chloride binding site is Arg¹⁰²². Addition of Ca²⁺ increases enzyme activity and stability (data not shown). Five residues may play a role in the

		◇ ↓ ∇ ∇		↓ ∇ ∇ ∇	
ASR	633	-	RVDAVDNVDADLLKIAGDYFKALYGTDKSDANANKHLSILEDWNG	-	677
GTFD	463	-	RVDAVDNVDNADLLQIASDYKKAHYGVDKSEKNAINHLSILEAWSD	-	507
DSRS	549	-	RVDAVDNVDADLLQIAADYFKLAYGVDQNDATANQHLSILEDWSH	-	593
GTFA	1022	-	RVDA <u>PDNI</u> ADL <u>MN</u> IAQDYFNAA YGMD-SDAVSNKHINILEDWNH	-	1065
			**** *: : *: : ** *: : ** * . : : *: : ** *		
AS	292	-	RMDAVAFIWKQMOT	-	305
					333-FKSEAIVH-340

FIG. 5. Alignment of parts of the catalytic cores of DSRS of *L. mesenteroides* NRRL B-512F (46), GTFD of *S. mutans* GS5 (15), ASR of *L. mesenteroides* NRRL B-1355 (3), GTFA of *L. reuteri* strain 121, and amylosucrase (AS) of *N. polysaccharea* (10). Amino acids which are conserved in all GTFs but not in GTFA are shown in boldface type (also underlined). Symbols: *, identical residue; :, highly conserved residue; ., conserved residue; ↓, putative catalytic residue; ∇, residue possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ◇, putative chloride binding site. The alignment shown is based on an alignment of glucansucrases made by Monchois et al. (30).

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1      1515-----IYNLPCKEVA--TVTRV
2      1542---NNNLVYVNTIGGGEY--QKKYG
3      1571--LYPEIFTKQVSTGVAIDPSQKITE
4      1597-----SAKYFN--GTNILLHRGSGYV
5      1618---DGGQYYNL-GTTTK--QFLPI
6      1649--GNDGNYYFDLAGNMVK--NTFIE
7      1673--DSVGNWYFFDQDGKME--NKHFV
8      1700--GEKGTYYFLKNGVSR--GGLVQ
9      1721--TDNGTYFFDNYGKMVR--NOTIN
10     1743---GAMIYTLDENCKLTK--ASYSN
11     1763---DAEYPTSTDVCKMLD--QNKLI-

```

FIG. 6. Alignment of repeats in the C-terminal region of GTFA of *L. reuteri* strain 121. YG repeats consist of, from left to right, (i) four to six residues that usually include glycine and/or aspartate and/or asparagine residues (indicated in boldface type), (ii) a cluster of one to four residues (lightly shaded) that nearly always includes tyrosine (underlined), (iii) three to four poorly conserved residues, (iv) a glycine residue (boxed), (v) two poorly conserved residues, (vi) a residue that is usually hydrophobic (indicated in boldface italic type and lightly shaded), (vii) one poorly conserved residue, (viii) a neutral polar residue (darkly shaded) that is usually glycine or asparagine (indicated in bold type), (ix) one poorly conserved residue, and (x) three residues (darkly shaded) that include one to three hydrophobic residues (underlined) (19). Four repeats (rows 6, 7, 8, and 9) match exactly the consensus sequence defined by Giffard and Jacques (19). The other seven show lower homology with the YG repeats.

binding of acceptor molecules and the transfer of the glucosyl residue. These are GTFA residues Asp¹⁰²⁷, Asn¹⁰²⁸, Asp¹⁰⁶², and Trp¹⁰⁶³ (Fig. 5). The fifth amino acid, possibly playing a role in acceptor binding or transfer of the glucosyl residue, a Ser in other GTFs (except for ASR of *L. mesenteroides* and DSRA of *L. mesenteroides*), was replaced by Asn¹⁰⁶⁴ in GTFA.

The C-terminal domain of GTFA, which consists of 267

amino acids, is shorter than corresponding domains in other GTFs (~500 amino acids). The C-terminal domain of streptococcal and *Leuconostoc* GTFs consist of a series of different tandem repeats, which have been divided into four classes: A, B, C, and D repeats. These repeats exhibit high similarity to the repeats found in the glucan binding protein from *S. mutans* as well as the ligand binding domains in *Clostridium difficile* toxin A and the lysins from *Streptococcus pneumoniae* (18, 47). DSRS from *L. mesenteroides* NRRL B-512F also contains, in addition to A and C repeats, N repeats, which have not been identified in streptococcal GTFs. ASR from *L. mesenteroides* NRRL B-1355 contains a single A repeat and distinct short repeats DG(X)₄APY (24). Within the A, B, C, and D repeats, a repeating unit designated YG can be distinguished (19). The A, B, C, and D repeats present in distinct patterns in the C-terminal domain of other GTFs were not found in GTFA. Instead, four YG repeating units and seven less-conserved YG repeats could be identified (Fig. 6).

The highest overall homology of GTFA at the amino acid level was found with ASR from *L. mesenteroides* NRRL B-1355 (3), which is responsible for the synthesis of an alternan with 50% α -(1→6) and 50% α -(1→3) linkages, and with DSRS from *L. mesenteroides* NRRL B-512F (29, 46), which synthesizes a dextran with 95% α -(1→6) and 5% α -(1→3) linkages. Homology of GTFA with other GTFs was highest in the highly conserved putative catalytic domain, which had roughly the same size and structure as the corresponding domains of other GTFs. However, not all the conserved residues were found in the *L. reuteri* strain 121 GTFA. Relatively many differences with amino acids conserved in other GTFs were found directly downstream of the putative catalytic Asp¹⁰²⁴ (Fig. 5). This region constitutes the α/β -barrel 4 of the enzymes of family 13

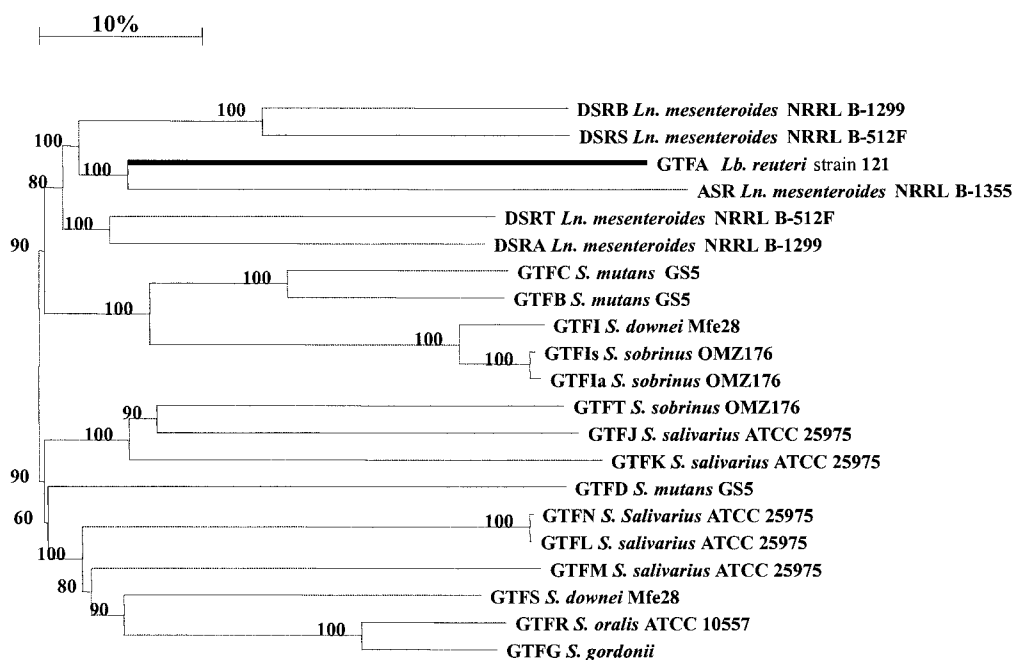


FIG. 7. Dendrogram of GTFs of lactic acid bacteria. The horizontal distances are a measure for the differences at the amino acid level. The length of the upper bar indicates a 10% difference. Bootstrap values are given at the root of each branch (as a percentage). The GTFA of *L. reuteri* strain 121 is indicated with a thick black line.

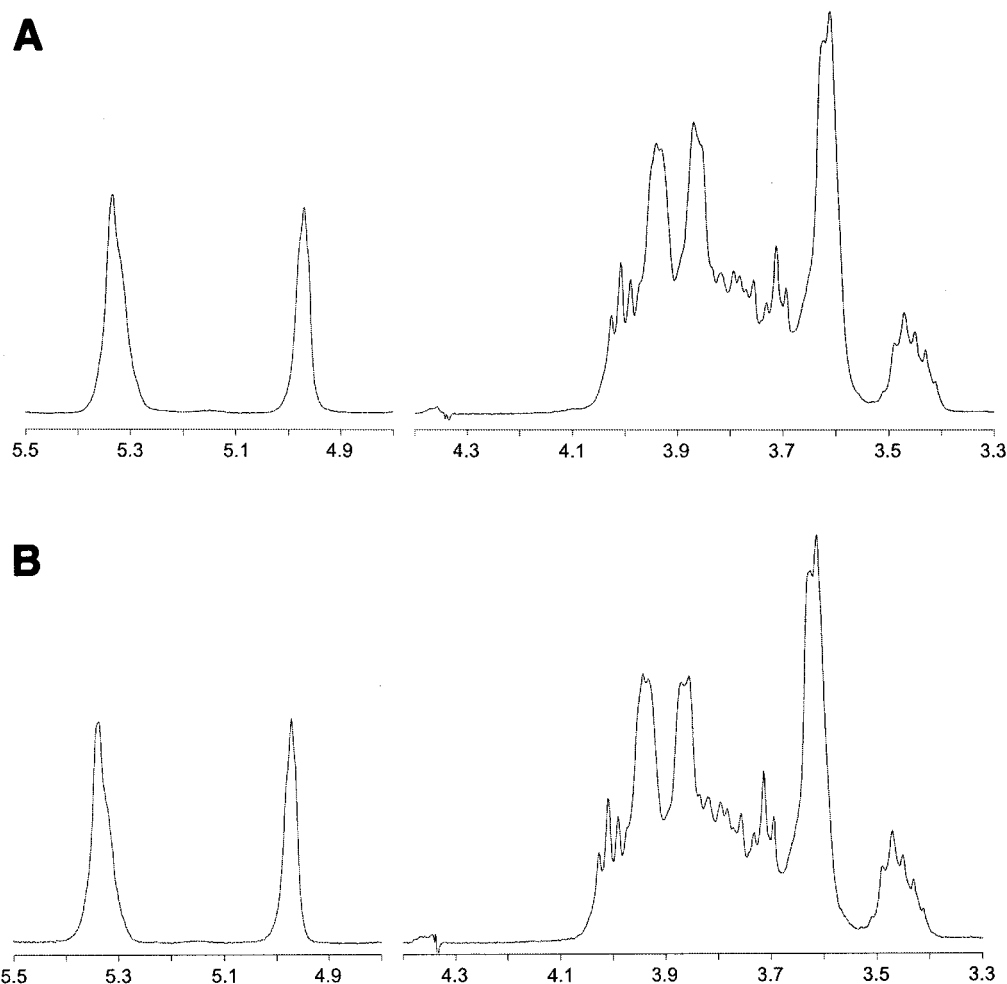


FIG. 8. 500-MHz ^1H NMR spectra of the glucan produced by *L. reuteri* strain 35-5 GTFA present in culture supernatant (A) and by *E. coli* GTFA cell extracts (B), recorded in D_2O at 80°C . Chemical shifts are given in parts per million relative to the signal of internal acetone ($\delta = 2.225$).

of glycoside hydrolases (27). The domain directly downstream of the catalytic Asp¹⁰²⁴ contains the conserved amino acids Asp-Ala-Val-Asp-Asn in other GTFs. In CGTase these residues constitute part of the acceptor binding site (residues Asp²²⁹-Ala-Val-Lys-His²³³ in *Bacillus circulans* 251 CGTase), responsible for the stereospecific positioning of the molecule accepting the glucosyl unit (25). The structure of this acceptor site determines the type of glucosidic bond formed (41). In the corresponding region of GTFA, Pro¹⁰²⁶ is found in a position where a conserved Val is found in other GTFs (Fig. 5). Com-

pared with Val, the presence of Pro causes a more rigid protein structure, which may have a direct effect on the type of glucosidic bonds formed in the glucan synthesized by the enzyme. The presence of the Pro¹⁰²⁶ residue could therefore be part of the explanation for the unique structure of the glucan with α -(1 \rightarrow 4) and α -(1 \rightarrow 6) bonds produced by GTFA. The conserved Val is present in amylosucrase (Fig. 5), a GTF synthesizing α -(1 \rightarrow 4) bonds. However, immediately downstream of this Val, the conserved Asp-Asn residues are replaced by Ala-Phe (10). The following amino acid in amylosucrase is Ile, which is also present at that position in GTFA, whereas in other GTFs a conserved Val is found (Fig. 5). This also suggests that the above-mentioned region downstream of the catalytic Asp¹⁰²⁴ may be of influence on the type of bonds being formed. Therefore, Pro¹⁰²⁶ and Ile¹⁰²⁹ of GTFA are likely targets for site-directed mutagenesis experiments.

The partial ORF upstream of *gtfA* (ORF2 [Fig. 1]) may encode a second GTF enzyme in *L. reuteri* strain 121. However, the N-terminal amino acid sequence of GTF purified from culture supernatants of *L. reuteri* strain 121 was the same as the deduced N-terminal amino acid sequence of the *gtfA* gene, and

TABLE 1. Methylation analysis of the glucans produced by *L. reuteri* strains and *E. coli* GTFA

Type of glucosyl unit	% Methylation		<i>E. coli</i> (pBGTf1)
	<i>L. reuteri</i> strain 121	<i>L. reuteri</i> strain 35-5	
Glc _p -(1 \rightarrow	24	25	21
\rightarrow 4)-Glc _p -(1 \rightarrow	42	43	44
\rightarrow 6)-Glc _p -(1 \rightarrow	22	21	24
\rightarrow 4,6)-Glc _p -(1 \rightarrow	12	11	11

the M_r and pI of the purified enzyme were the same as those predicted from the nucleotide sequence of the *gtfA* gene. Furthermore, the ^1H NMR spectra of the glucans produced by the *L. reuteri* GTFA present in culture supernatant and by the *E. coli* GTFA in cell extracts were virtually identical (Fig. 8). This, combined with the results of the methylation (Table 1) and the molecular weight determinations of the glucans, shows that the *E. coli* GTFA and the *L. reuteri* enzyme present in culture supernatants synthesize the same glucan with a unique structure: a highly branched glucan containing α -(1 \rightarrow 4) and α -(1 \rightarrow 6) bonds. Therefore, it is concluded that the *gtfA* gene encodes the active GTF of *L. reuteri* strain 121.

In the future, ORF2 upstream of *gtfA* will be characterized in further detail and GTFA will be characterized molecularly and biochemically.

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S.K. and G.H.V.G.-S. contributed equally to this work.

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